Resistance Allele Frequency to Bt Cotton in Field Populations of *Helicoverpa armigera* (Lepidoptera: Noctuidae) in China

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ABSTRACT Resistance evolution in target insects to *Bacillus thuringiensis* (Bt) cotton, *Gossypium hirsutum* L., is a main threat to Bt cotton technology. An increasing trend of population density of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) has been observed since 2001 in Qiuxian County (Hebei, China), where Bt cotton has been planted dominantly since 1998. This region was selected in 2006 and 2007 for estimating frequency of gene alleles conferring resistance to Bt cotton by screening the F_1 progeny from single-pair cross between field-collected male and laboratory female of the Bt-resistant strain of *H. armigera* (F_1 screen). F_1 offspring from each single-pair line were screened for resistance alleles based on larval growth, development, and survival on Bt cotton leaves for 5 d. Two-year results indicated that ≈20% of field-collected males carried resistance alleles. The conservative estimate of the resistance allele frequency was 0.094 (95% CI, 0.044–0.145) for 2006 and 0.107 (95% CI, 0.055–0.159) for 2007. This is the first report of resistance allele frequency increase to such a high level in the field in China. Long-term adoption of Bt sprays, dominant planting of single-toxin–producing Bt cotton, and lack of conventional cotton refuge system might accelerate the resistance evolution in the region.

KEY WORDS *Helicoverpa armigera*, Bt cotton, resistance detection, F_1 screen, resistance management

Helicoverpa armigera (Hübner) (Lepidoptera: Noctuidae) is one of the economically most important insect pests on cotton, *Gossypium hirsutum* L., in China. Control of this insect with conventional insecticides became more difficult because of resistance evolution to various chemical insecticides (Shen and Wu 1995, Forrester et al. 1993). Transgenic cotton, expressing the Cry1Ac toxin from *Bacillus thuringiensis* (Bt) has become one of the most important tools for managing the insect in China since 1997. Bt cotton growing area in China has rapidly expanded to 3.5 million ha, accounting for ≈66% of the total cotton area in 2006 (James 2006).

The wide adoption of transgenic Bt crops places a high selection pressure on the target insect populations and accelerates development of resistance (Huang 2006). At present, laboratory strains of more than a dozen insect species, including *H. armigera*, have been selected for Bt resistance (Tabashnik 1994, Ferré and Van Rie 2002, Meng et al. 2004, Griffitts and Aroian 2005). However, field-evolved resistance to Bt crops has not been reported yet (Tabashnik et al. 2003, Heckel et al. 2007). The development of effective monitoring system for implementation of effective resistance management (IRM) plans has become critical to ensure the long-term durability of Bt plants (U.S. EPA and USDA 1999).

Many Bt resistance monitoring methods have been proposed to detect early shifts in Bt-resistant allele frequencies in field populations (Huang 2006), including dose response (Wu et al. 2006, Wu 2007), diagnostic dose (Roush and Miller 1986), in-field screen (Venette et al. 2000), F_2 screen (Andow et al. 1998), and screening the F_1 offspring from single-pair crosses between field-collected male and laboratory female of the Bt-resistant strain of *H. armigera* (Gould et al. 1997), which is named as F_1 screen in this study. Because high levels of resistance to Bt toxins and Bt crops are often encoded by recessive or partially recessive alleles (Akhurst et al. 2003, Tabashnik et al. 2005, Xu et al. 2005), it is difficult to monitor early shifts of heterozygous allele frequencies by using the LC_{50} and other dose–response parameters, which are more suitable for detecting homozygous resistant individuals in large proportion. The strengths of the F_1 screen include the ability to detect major recessive resistance alleles and to detect the resistance alleles not only at the same loci as r allele in the resistant strain but also at the other loci if they confer resistance

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and had dominant expression in field populations (Gould et al. 1997). Therefore, F_1 screen and F_2 screen methods are especially effective and sensitive bioassay methods in detecting rare resistance alleles at early stage of resistance evolution. Compared with the F_2 screen, the F_1 screen technique is more efficient and saves time and cost. However, establishment of a highly resistant strain is the key for developing the F_1 screen technique.

In our laboratory, the YCR strain of *H. armgiera*, selected with Bt cotton leaves, developed a very high level of resistance to Cry1Ac protoxin (≈1,680–1,780-fold) after 42 generations of selection (Meng et al. 2004). The resistance was further increased to >7,000-fold after 88 generations of selection (unpublished data). Genetic crosses demonstrated that the resistance in the YCR strain of *H. armigera* was controlled by a major autosomal, incompletely recessive gene (Zhou and Shen 2005).

The current strategy for delaying the evolution of resistance to Bt crops in target insects is known as high dose plus refuge strategies (Carrière et al. 2005; Tabashnik et al. 2005). The three most important prerequisites for a successful refuge strategy are "high dose" expression of Cry1Ac in Bt cotton, "non-Bt crop refuge," and "rare resistant allele frequency" in field populations. The U.S. EPA requires that each farm set aside some (≈5%) land for non-Bt cotton. In Australia, the government and farmer groups have decided to restrict Bt cotton to 30% of the land, leaving a large refuge for susceptible bollworms (Gould et al. 2002). No mandatory IRM plans are required for planting Bt cotton in China. A natural refuge, so named as unintentionally mixed planting of cotton, corn (*Zea mays* L.), soybean [*Glycine max* (L.) Merr.], peanut (*Arachis* spp.), and other hosts of the pest on single-family farms of a small scale (<1 ha) to form a mosaic pattern over a large cotton growing area, has been adopted in Bt cotton growing areas for delaying resistance evolution. This natural refuge system was considered functionally similar to the mandatory refuges for large cotton farms in western countries for managing resistance evolution to Bt cotton (Wu et al., 2005). Consequently, not only the cropping system in China is quite different from the large-scale farming in the United States and Australia but also the proportion of natural refuges from other crops is variable in the different Bt cotton-growing areas. It has been reported that the host plants of *H. armigera* were planted on ≈8 million ha farmland in proportions of 26, 32, 28, and 13% for cotton, corn, peanut, and soybean, respectively (China Agricultural Yearbook 2006).

Unlike in other cotton growing areas in China, cotton is preferred crop in Qiuxian County (Hebei, China). Currently, complete Bt cotton is adopted in the area, with ≈68% of the total farmland planted with Bt cotton. Field surveys indicated that the population density of the bollworm increased 3–20-fold in the region between 2003 and 2007 (unpublished data). Wan et al. (2005) reported that ≈5–20% of naturally occurring larvae survived on Bt cotton in late growing season. To evaluate effect of natural refuge strategy and more importantly, to provide a basis for the timely implementation of new management tactics to control resistant populations, we used the F_1 screen method described by Gould et al. (1997) to directly estimate the frequency of alleles conferring resistance to Bt cotton in *H. armigera* in 2006 and 2007.

Materials and Methods

Insects. The susceptible strain (YCS), collected originally from a cotton fields near Yanshi City (Henan, China) in July 1991, has been reared for 145 generations on artificial diet without exposure to any insecticides including Bt toxins. To maintain the susceptibility to Bt, the YCS strain was purified once a year by using single-mating line. This strain was used in this study as reference for comparison of larval growth rate.

The resistant strain (YCR) was developed from a population originally collected from a cotton field near Yanshi City. Considering Bt protein expressed in Bt plants can be different from the endotoxins produced by the *B. thuringiensis* (National Research Council 2002), Bt cotton leaves ($R_{19}/33^B$ expressing Cry1Ac toxins), instead of laboratory produced Bt toxin, were used to continuously select the colony for 91 and 102 generations, which were used for screening tests in 2006 and 2007, respectively.

Field populations were collected in 2006 from Nanliu village and repeated in 2007 from Boliugu village. The two villages, ≈8 km apart, are located in Qiuxian County (Hebei, China). Male moths of the second field generation were collected using two light traps from 16 June to 21 June in both 2006 and 2007. Two traps were set >2 km apart, and each trap covered a large open area of the Bt cotton field in Qiuxian County (Hebei, China). The field-collected male moths were transferred to laboratory, and they were allowed to individually (single-pair) mate with the virgin female moths of the YCR strain. The F_1 progeny were used for F_1 screen.

Transgenic Bt Cotton. For all tests, the Bt cotton Xinmian33^B (common name is NuCOTN33^B), a commercial variety expressing Bt insecticidal crystal protein Cry1Ac, was purchased from Monsanto Far East Ltd. (Beijing, China). The leaves for growth rate experiment and F_1 screen were collected from the plants at seedling stage (6–7 wk old) grown in a clear-roofed greenhouse. Bt Cry1Ac toxin expression in Bt cotton was verified using the YCS strain as described by Meng et al. (2000), and only the plants producing sufficient toxin to kill all susceptible *H. armigera* were used for bioassay experiments. The non-Bt conventional cotton, SM-12 provided by Tai Cang Elite Seed Station (Jiangsu, China), was used as the control.

Growth Rate of *H. armigera* on Bt Cotton. Growth rate experiment was conducted to distinguish homogenous resistant genotype r_1r_1 from heterozygous genotype r_1s in *H. armigera* larvae based on their growth rate on Bt cotton leaves for 5 d. The top second or third expanded tender leaves of 6–7-wk-old Bt cotton were removed, and the leaf stem was wrapped with mois-

ture cotton. The leaf was individually placed into a 250-ml clear plastic cup. Five neonates of each of the laboratory strains, the resistant YCR strain, the susceptible YCS strain, and the hybrids of reciprocal crosses F_1 ($♀_{YCR} \times ♂_{YCS}$) and F_1' ($♀_{YCS} \times ♂_{YCR}$), were transferred, respectively, onto the cotton leaf within the plastic cup with a fine brush. Plastic membrane and black cloth were used to cover the cups to prevent the larvae from escaping. The larvae were kept at 28 ± 1°C, 70–80% RH, and under a photoperiod of 14:10 (L:D) h for 5 d. Each replicate contained five cups and four replicates (total 100 neonates) were used for each treatment (strain). After 5 d, the survival rate, developmental stage, and larval body weight of all survivors were recorded. The developmental stage (instar) was determined based on head capsule and body size. Because larvae could not reach third instar on Bt cotton leaf in 5 d; therefore, individual larval growth and development were not influenced by grouping larvae together within the cup. For control, non-Bt cotton leave were used to assay growth rate of YCR, YCS, and hybrids from reciprocal crosses under the same conditions.

To compare the growth and survival rates of the susceptible reference strain (YCS) with field population of *H. armigera* on Bt cotton, we obtained a few isolines of *H. armigera* directly from field-collected females in 2007, and tested their offspring on Bt cotton leaf for 5 d by using same method and conditions described above.

F_1 Screening for Resistance Genotype. To estimate the resistant allele frequency in field populations of *H. armigera*, we used an improved F_1 screen method originally developed by Gould et al. (1997). In brief, field-collected males (ss, rs, or rr genotype) were individually mated to virgin females (r_1r_1) of homozygous resistance strain (YCR). Their progeny (either homozygous [i.e., rr_1] or heterozygous [i.e., sr_1]) were tested on Bt cotton leaves by using the same method of growth rate assay. Theoretically, homozygous susceptible male and homozygous resistant female produce only heterozygotes, which are susceptible and unable to grow on Bt cotton leaves because of the recessive nature of the resistance. If the males carry homologous resistant alleles (rr), their progeny will survive on Bt cotton leaves because they inherited an r_1 allele from their mother and a field-derived resistance allele r from their father. If a male carries both r and s alleles, ≈50% of their progeny will survive on Bt cotton. Based on this assumption, we can infer whether the male carried the resistance allele. Therefore, F_1 survivors were considered resistant individuals if they reached the same growth rate of the resistant strain on Bt cotton leaves for 5 d.

Male moths were collected using light traps from Bt cotton-growing areas. Each male was allowed to mate with a virgin female of the YCR strain. To prevent potential contamination of male pupa during the process of sexing the pupae, female pupae of the YCR strain were set as groups. The groups once containing any male moth were discarded, and the groups of pure females were used for single-pair mating with field-collected males. Single pair was placed in a 250-ml clear plastic cup covered with cheesecloth to provide a substrate for egg laying. Moisture was supplied with a moistened cotton pad, and 4% sugar solution was provided for food. Paired moths were maintained at 28 ± 1°C, 70–80% RH, and a photoperiod of 14:10 (L:D) h. Eggs from each single mating pair were sanitized in 5% formaldehyde formamide solution for 3 min to prevent pathogen contamination. Approximately 100–200 neonates of F_1 generation from each single-mating pair were tested using excised Bt cotton leaves following the same procedures for the growth rate assay. After 5 d, survivors were scored for developmental stage, and their weight was measured. If the survivors grew and developed at same rates as the resistant strain, they were considered as resistant, and their wild male parent was considered to carry the major resistant allele. The potential positive lines from F_1 selection were further verified with F_2 screening as described below.

F_2 Rescreening for Eliminating False Positive Families. To eliminating false positive lines, all potential F_1 positive lines were rescreened. The F_1 survivors were removed from Bt cotton leaves after 5 d and weighed. These putative resistant larvae (reached growth rate of resistant strain) were transferred to glass test tubes supplied with artificial diet and then individually reared to pupation. After moths emerged, single-sib-mating pairs were set up for all potential positive F_1 families. Approximately 100 F_2 neonates of each F_1 sing-pairs were screened on Bt cotton leaves by using the same method for growth rate assay. After 5 d, the survival and the developmental rates were recorded.

Data Analysis. Data were statistically analyzed using SAS program (SAS Institute 1990). Proc GLM and Proc REG procedures were used for variance and regression analyses. Mean separation was conducted using SAS Proc Means/least significant difference (LSD) or Lsmeans separation programs at $P < 0.05$.

Results

Differential Growth Rates of Susceptible, Resistant, and Field Populations. In total, 100 neonates from each of the parent strains (YCR and YCS), F_1 and F_1' progeny from reciprocal crosses were reared on Bt cotton leaves. After 5 d, overall survival rate of YCR strain was 75 ± 1.9% (Fig. 1A). Among these YCR survivors, 63.0 ± 6.1% of the larvae reached body weight ≥0.8 mg, 24.0 ± 4.5% of the larvae had body weight of ≈0.6–0.7 mg, and 13.0 ± 5.2% of the larvae had body weight of ≈0.4–0.5 mg. The overall survival rate of F_1 ($♀_{YCR} \times ♂_{YCS}$) was 6 ± 1.2%. Two of six survivors had body weight of 0.4–0.5 mg, and the others reached only 0.1 to 0.3 mg. F_1' ($♀_{YCS} \times ♂_{YCR}$) only had two (2 ± 1.2%) survivors and their body weight ranged 0.4–0.5 mg. No larvae in the YCS strain survived.

The results indicated that Bt-susceptible and Bt-resistant *H. armigera* had significant difference in larval survival rate ($F_{1,15} = 1458.51$; $P < 0.0001$) and growth rate ($F_{1,82} = 23.93$; $P < 0.0001$) on Bt cotton

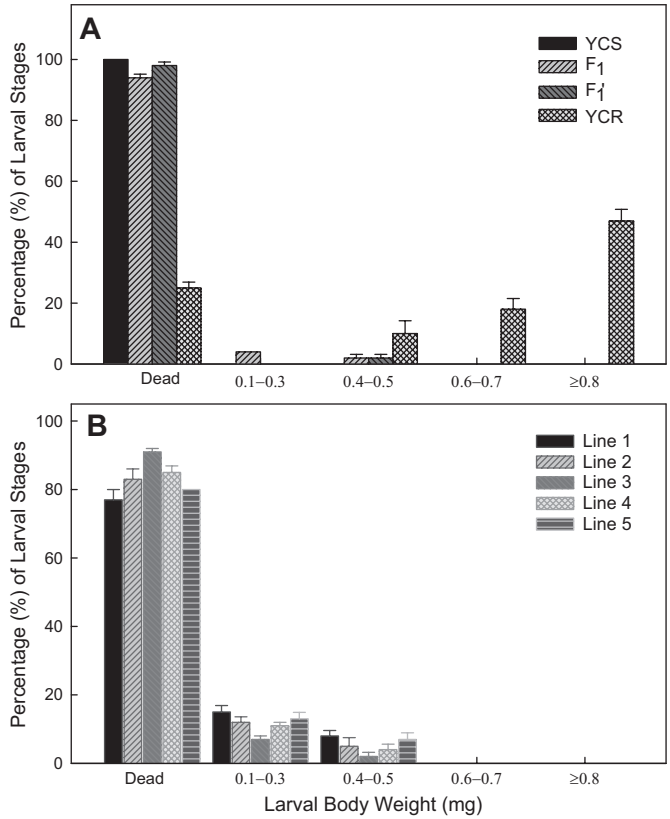

Fig. 1. Histograms showing the percentages of larvae that reached different stages after 5-d feeding on Bt cotton. (A) YCS, susceptible strain; YCR, resistant strain; F_1, hybrids ($♀_{YCR} \times ♂_{YCS}$), F_1', hybrids ($♀_{YCS} \times ♂_{YCR}$). (B) Lines 1–5 were progeny from five field-collected females.

leaves. The YCS susceptible strain (ss) was unable to survive on Bt cotton. Most heterozygous individuals (r_1s) died after feeding Bt cotton, and only 2–6% heterozygotes survived, but the survivors could not reach body weight of ≥0.6 mg and developmental stage of mid-second instar. Whereas resistant individuals (r_1r_1) had considerably higher survival rate (75%), and a majority of survivors (65% of the total or 87% of the survivors) reached ≥0.6 mg and at least mid-second instar. Therefore, individuals, reached ≥0.6 mg and beyond mid-second instar on Bt cotton leaves for 5 d, were categorized as resistant individuals. However, not all resistant larvae survived on Bt cotton for 5 d as expected, possibly due to some adverse factors such as fitness cost, incomplete resistance, or other heredity traits. Only 75% resistant larvae survived after 5-d feeding on Bt cotton leaves, and the larvae with reduced weight (≤0.5 mg) also were observed in the resistant strain. In addition, 12% resistant larvae also died on non-Bt cotton. The corrected survival rate for resistant strain on Bt cotton should be 85.2% (uncorrected 75%).

To compare growth and survival rates of field population with those of laboratory strains and hybrids, neonates derived from each of five field-collected females were subjected to Bt cotton selection for 5 d. Survival rates for each field line were 9, 15, 17, 20, and 23%, all of which were higher than that of YCS, F_1, or F_1'. However, all survivors could not exceed mid-second instar and their body weight could not reach ≥0.6 mg (Fig. 1B).

F_1 Screening by Using Bt Cotton Leaves. In 2006, 353 single-mating pairs in total were established from field-collected male moths, and only 127 (36.0%) pairs successfully laid sufficient fertile eggs to enable F_1 screen. The loss of other mating pairs was mainly due to unsuccessful mating of the field-collected males with laboratory resistant females, possibly associated with low mating preference and limited sperms in field-collected males (Stodola et al. 2006). Approximately 159.4 ± 7.3 (mean ± SE) F_1 neonates per single-pair family were assayed on tender Bt cotton leaves for 5 d. Among the 127 families assayed, various numbers of F_1 progeny survived in 122 lines, whereas all larvae from the other five lines died after feeding on Bt cotton for 5 d. Survival rates for all 122 lines ranged from 0.7 to 40%. In total, 2,816 larvae survived the 5-d treatment on Bt cotton. Larval body mass ranged from 0.1 to 2 mg. Approximately 85% survivors had body weight <0.6 mg. Regression analyses indicated a correlation between survival rate and larval body weight for each line (Fig. 2A1 and A2). The R^2

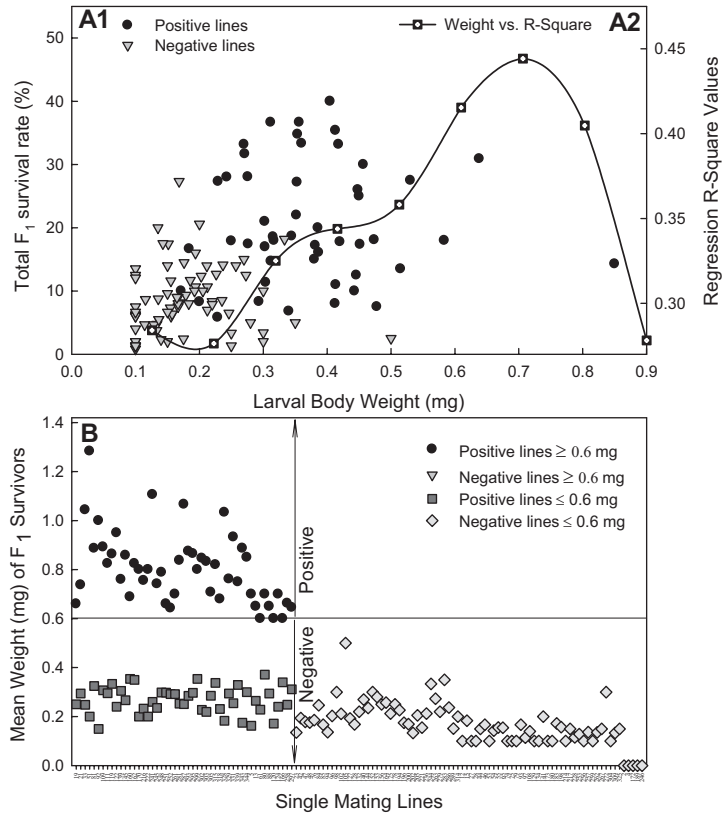

Fig. 2. Relationship between larval body weight (mg) and total survival rate established from 2006 F_1 screen on Bt cotton for 5 d. (A1) Scatter plot of total larval survival rate against larval mean weight of all larvae ≥0.1 mg. (A2) Regression curve constructed by plotting the R^2 values against mean body weight including different weight range, i.e., 0.1 mg larvae included all larvae with body weight ≥0.1 mg, 0.2 mg larvae included all larvae with body weight ≥0.2 mg, and so on. (B) ● and ▼, weight of positive and negative lines, respectively, averaged over the larvae ≥0.6 mg after 5-d feeding on Bt cotton. All positive lines had a mean body weight ≥0.6 mg, and no larvae in negative lines had a mean body weigh ≥0.6 mg; ■ and ♦, weight of positive and negative lines averaged over the larvae ≤0.6 mg.

value reached the maximum as the body weight increased to 0.7 mg and higher ($P < 0.001$; Fig. 2A2); however, the correlation slope decreased as the weight was beyond 0.6 mg. To minimize underestimation of resistance alleles, both larval body weight of ≥0.6 mg and developmental stage of mid-second instars were used as a reference for differentiating positive (putative resistant) from negative (putative susceptible) lines. Among 122 surviving lines, 49 lines reached body weight ≥0.6 mg and developed beyond mid-second instar. No one in the other 73 lines had body weight >0.5 mg. Average weight for the 73 lines was 0.18 ± 0.01 mg, comparing with the mean weight (0.27 ± 0.01 mg) of the larvae with corresponding weight range (≤0.6 mg) in the 49 lines (Fig. 2B). The growth and development rates of the 49 lines were similar to those of resistant strain, and then these lines were considered as potential positive lines with YCR-like resistance gene alleles. Other 73 lines did not grow and develop as fast as the resistant strain, and then were considered as negative lines or susceptible lines.

In 2007, 374 males in total were collected and mated to YCR virgin females. Of the field-collected males, 135 (36.1%) mating pairs successfully laid sufficient fertile eggs for F_1 screening. To minimize experimental errors, ≈165.2 ± 5.2 F_1 neonates per line were tested for their susceptibility to Bt cotton. Two lines died completely. Survival rates for all 133 lines ranged from 3 to 51.8%. In total, 4,966 larvae survived 5-d feeding on Bt cotton. Larval body mass ranged from 0.1 to 2.3 mg. Approximate 85% survivors had body weight <0.6 mg. Regression analyses (Fig. 3A1 and A2) indicated that the R^2 value reached the maximum as the body weight increased to 0.6 mg and higher ($P < 0.001$; Fig. 3A2). Larvae from 44 lines survived and reached the mid-second instar stage and a body weight ≥0.6 mg. No larvae in the other 89 surviving lines had a body weight >0.5 mg. Average weight for the 89 lines was 0.19 ± 0.01 mg, compared with the mean weight 0.29 ± 0.01 mg of the larvae with corresponding weight range (≤0.6 mg) in the 44 lines (Fig. 3B). These 44 lines were considered as potential positive lines. Other 91 lines, did not survive on Bt cotton or exhibited lower growth rate and slower development, were considered as negative lines.

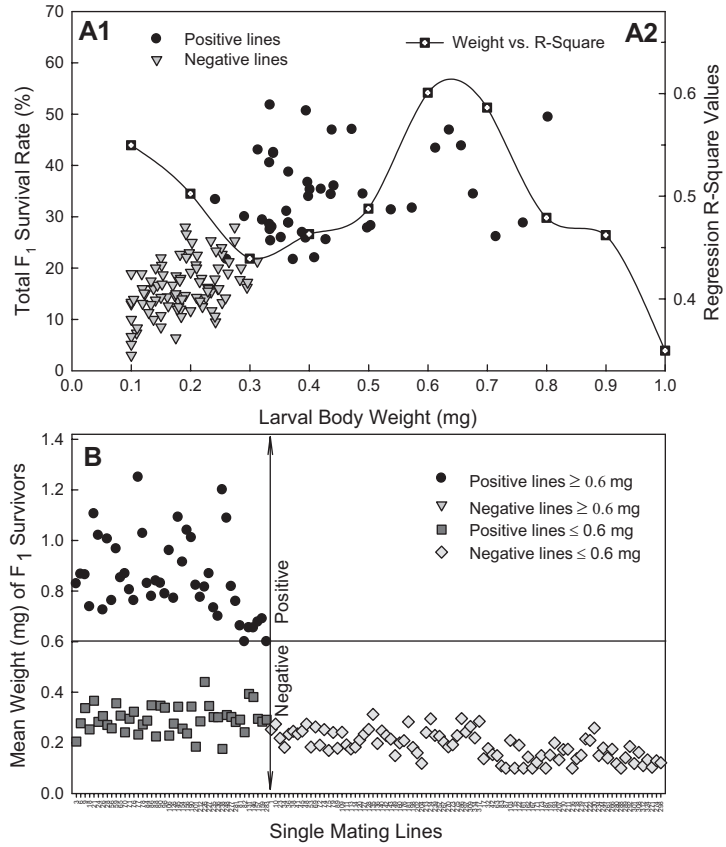

Fig. 3. Relationship between larval body weight (milligrams) and total survival rate established from 2007 F_1 screen on Bt cotton for 5 d. See Fig. 2 legend for details on A1, A2, and B.

F_2 Verification. If these F_1 larvae reached ≥0.6 mg weight and at least mid-second instar after feeding on Bt cotton, it was likely that the survivors carried rr_1 genotype obtained from wild male (rr or sr) and resistant female (r_1r_1) parents. After sib-mating, F_2 offspring were expected to have rr, rr_1, and r_1r_1 genotypes. Theoretically, all F_2 larvae can survival on Bt cotton. However, when the single-pair mating happened between a moth with r_1s genotype (developed from large larva in a few case, or due to partial recessiveness of the resistance) and a moth with r_1r genotype, ≈50% their progeny can survival on Bt cotton. In a few other occasional cases, wild males might have an ss genotype. Their heterozygous progeny (r_1s) might reach body weight ≥0.6 mg and at least mid-second instar due to some noninheritable factors or having different resistance gene(s) compared with YCR strain. After sib-mating, ≈25% of F_2 offspring might carry r_1r_1 and 50% of F_2 offspring might carry r_1s genotypes, then ≈25% F_2 progeny will survive on Bt cotton. Because the resistant strain had 85.2% (instead of 100%) of corrected survival rate on Bt cotton, corresponding survival rate for 25% r_1r_1 F_2 offspring would be ≈21.3% (instead of 25%). Meanwhile, ≈2–6% of heterozygotes ($F_{1♀[YCR] \times ♂[YCS]}$ and $F'_{1♀[YCS] \times ♂[YCR]}$) could survive on Bt cotton (Fig. 1), and 50% of the r_1s genotypes would add additional 2% of survivors to make a total of 23.3% potential survival rate in F_2 offspring. Nevertheless, to minimize overestimate, the F_2 offspring with maximal survival rate up to 28% (25% r_1r_1 genotype plus 3% maximal survival rate of 50% sr_1 genotype derived from field male with ss genotype) were classified as false positive lines. Considering not all of resistant individuals can survive on Bt cotton leaves, caused partially by survival costs and incomplete recessive resistance, F_2 offspring with survival rate >28% (instead of 50% or 43% after correction) were classified as true positive lines.

In 2006, F_2 progeny of 33 (of 49) mating pairs were selected with Bt cotton for 5 d, and other 16 either died in early stage or laid insufficient fertile eggs (Fig. 4A). An overall average of 90.6 ± 2.6 F_2 larvae were obtained and subjected to Bt cotton selection. Among the 33 single mating lines, nine lines (160, 162, 258, 261, 287, 295, 303, 315, and 318) showed that their F_2 neonates had 5–28% survival rates on Bt cotton. Because none of these nine lines had survival rate greater than the expected value of 28%, they were therefore considered to be false positive lines.

Among 33 putative positive lines from F_1 screening, 24 (Fig. 4A) single-pair families produced F_2 progeny,

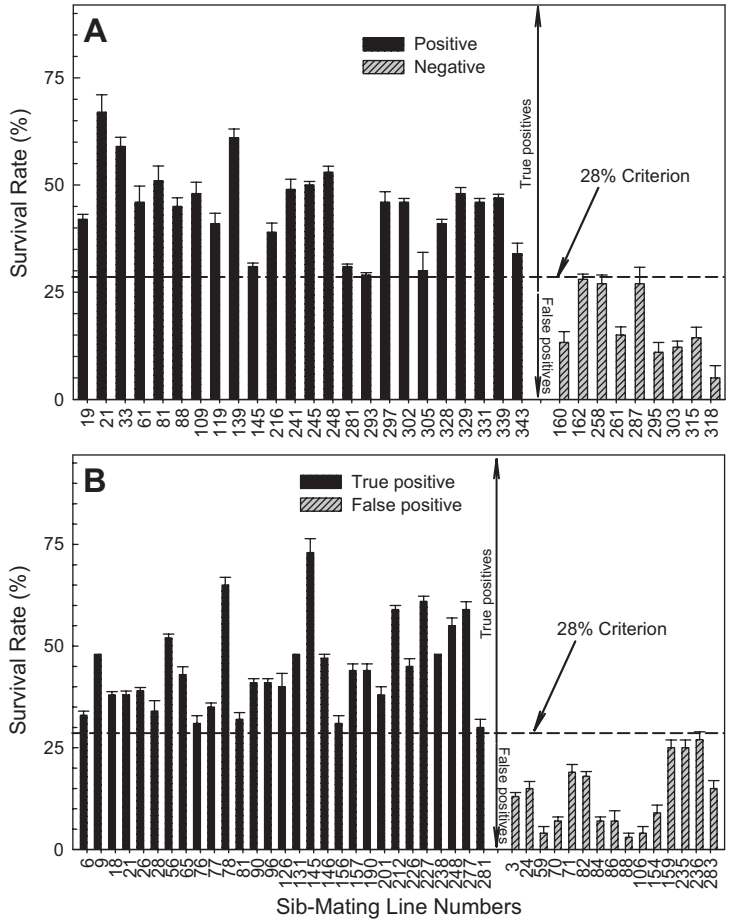

Fig. 4. Survival rates of F_2 progeny after rescreening on Bt cotton for 5 d. F_2 offspring with maximal survival rate up to 28% (25% r_1r_1 genotype plus 3% maximal survival rate of 50% sr_1 genotype derived from field male with ss genotype) were classified as false positive lines. (A) Rescreening of 33 putative positive lines in 2006. (B) Rescreening of 44 putative positive lines in 2007.

which were able to survive 5-d selection on Bt cotton and to reach growth rate and developmental stage as resistant strain. The survival rates of F_2 neonates on Bt cotton ranged from 29 to 67%. The survival rates were higher than the criterion of 28%, but they were lower than the expected value of 50 and 100% for the lines with heterozygous and homozygous parents, respectively. These 24 lines were therefore verified as positive resistant lines that may carry resistance gene alleles as the YCR strain.

In 2007, 44 putative positive lines were sib-mated and produced an average of 101.0 ± 1.4 F_2 neonates per F_1 single-pair for F_2 resistance verification screening on Bt cotton. Twenty-nine lines showed that their progeny were able to reach a growth rate and developmental stage as resistant strain on Bt cotton. Survival rates for these lines ranged from 30 to 73% after 5-d feeding on Bt cotton. The rates were lower than theoretical positive survival rates (50 and 100%), possibly due to fitness cost, but higher than the negative maximal survival rate (28%). Therefore, they were confirmed as true positive lines. The other 15 lines (survival rates ranged from 3 to 27%) were false positive lines because their survival rates were not greater than the criterion of 28% (Fig. 4B).

Frequency of Resistance Alleles. Because the males are diploid, estimated frequency of resistance alleles in 2006 was 0.094 (24/[127 × 2]), with 95% confidence intervals ranging from 0.044 to 0.145 in the region. The expected resistance allele frequency in 2007 was 0.107 with a 95% CI between 0.055 and 0.159 (Table 1).

Discussion

Resistance is a major concern to the sustainability of Bt cotton technology for suppressing *H. armigera* population in China and many other counties. Most previous studies relied on Cry1Ac toxin for resistance monitoring and for an establishment of Bt resistant strain through laboratory selection (Xu et al. 2005). Although considerable research on Bt-cotton resistance in *H. armigera* have been done in major cotton-producing countries, including China, Australia, and India (Gujar 2005, Downes et al. 2007, Gujar et al.

Table 1. Estimates of resistance allele frequency to Bt cotton in field population of *H. armigera* in Qiuxian County (Hebei, China) by using F_1 screen method

Yr	No. single mating-pairs	No. pairs with fertile eggs	No. F_1 neonate for F_1 screen (mean ± SE)	Total no. F_1 survivors	Putative positive lines from F_1 screen	No. F_2 neonate for F_2 retesting (mean ± SE)	No. true positive lines	Frequency of resistance allele	95% CI
2006	353	127	159.4 ± 7.3	2,816	49	90.6 ± 2.6	24	0.094	0.044–0.145
2007	374	135	165.2 ± 5.2	4,966	44	101.0 ± 1.4	29	0.107	0.055–0.159

2007, Wu 2007), comparison and analysis of resistance levels in different years and different countries were often difficult because different monitoring methods, different sources of Bt toxin, different background of insect control and resistance management in different regions, and genetic variability in the field populations, might contribute to the variation of resistance levels detected. In Australia, studies with F_0 screening and F_2 screening showed no major changes in resistance in field populations (Bird and Akhurst 2004, Downes et al. 2007). In a survey with Cry1Ac, *H. armigera* collected from Bt cotton fields had just twofold higher resistance ratio than those from non-Bt cotton fields in India (Gujar 2005). Based on traditional bioassays, field populations in China maintained susceptibility to the Cry1Ac protein from 1997 to 2004 (Wu et al. 2002, 2006), and they showed little increase in the frequency of major nonrecessive resistance genes in F_0 offspring of field-collected females from Xiajin County (Shandong, China) during 2002–2005 (Li et al., 2007). Substantial increase of resistance, however, was found in the bollworm only to Bt cotton. F_2 screening indicated that the frequency of resistance alleles to transgenic Bt cotton doubled from 0.033 (95% CI, 0–0.081) in 2000 (Bt cotton planted for 3 yr) to 0.0685 (95% CI, 0.006–0.131) in 2001 in Weixian County (Hebei, China) (Shen et al. 2004). In this study, F_1 screen was applied for first time to examine major resistance alleles in field populations collected from Qiuxian County (Hebei, China). It was the first time that resistant allele frequency in *H. armigera* reached such high level (≈0.1) in China. Resistance levels increased over years might be associated with increasing selection pressure due to increased Bt crop planting area over years.

F_1 screen was used in this study because this technique was proven to be effective and sensitive for detecting rare resistance alleles at early stage of resistance evolution (Gould et al. 1997, Huang 2006). The application of the technique requires a reliable screening method for F_1 progeny. Instead of using Cry1Ac toxin for F_1 screening and F_2 rescreening, Bt cotton leaves were used in this study to simulate field conditions for resistant allele screening. To successfully apply F_1 screen using Bt plant, it is important to ensure sufficient Bt protein expression in Bt plant to kill all susceptive individuals because Bt insecticidal protein expressions in transgenic cotton vary significantly under different environment, soil properties, and agronomic management (Zhou et al. 2005, Rochester 2006). Bt performance may also be affected by non-Bt physiological defense of the plant (Olsen et al. 2005). To minimize overestimation of resistant allele frequency, we verified Bt protein expression before starting the bioassays to ensure no susceptible larvae survived on the Bt cotton.

Another important consideration for F_1 screen is requirement of an accurate standard for judging whether F_1 survivors are resistant or susceptible. Because the r allele from the wild male could confer a different level of resistance from r_1 allele of the laboratory resistant female, a low survival rate and variation in larval growth rate on Bt cotton might be seen (Gould et al. 1997). This phenomenon is also associated with substantial negative fitness costs due to attainment of resistance to Cry1A toxins (Oppert et al. 2000, Bird et al. 2004, Carrière et al. 2006). The phenotypic separations would not follow the Mendelian separation in F_1 and F_2 generations; therefore, we used the growth rate parameter alone in F_1 generation to prevent losing or underestimating positive lines. But for F_2 verification screening, we considered fitness cost, along with the theoretical survival rate, to classify the lines as either the true or false positives. Our estimates of resistant gene frequencies were conservative, because some resistant larvae did not survive or reach body weight of ≥0.6 mg on Bt cotton leaves due to the fitness cost or different resistant level in field populations, and some potential positive lines could not be rescreened due to unexpected early mortality and low fecundity. In addition, increasing sample size (at least 100) for F_1 screening and F_2 rescreening may minimize some unnecessary errors.

In this study, we documented a higher frequency of resistance alleles to Bt cotton in a field population of *H. armigera* from Qiuxian County by using F_1 screening and F_2 rescreening in 2006 and 2007. The frequency of resistance alleles in this region was 0.094 (95% CI, 0.044–0.145) for 2006 and 0.107 (95% CI, 0.055–0.159) for 2007.

Because of recessiveness and low initial frequency of the Bt resistance, resistance levels in natural population may fluctuate or increase at a slow speed in the early stage of resistance evolution. Many factors, such as fitness costs associated with resistance, insect migration (gene flow), dominance of resistance, genetic variation, and gene drift in the field, might be contributed to the slow resistance evolution to Bt in insects. Our 2-yr data indicated that resistance gene frequency showed certain increase from 2006 to 2007. In addition, substantial increase of F_1 survival rate by 14.6% in 2007 (Table 1) suggested that the resistance in field population was potentially increasing over the 2-yr period. In Qiuxian County, Bt cotton has been planted for 10 yr, the mean resistance-allele frequency for the period 2006–2007 was 0.101 (95% CI = 0.065–

0.137), which means one in every 100 individuals (0.1×0.1) would be homozygous resistant insect. As most models of insect resistance evolution to insecticides indicate that after resistance allele frequencies reaches 0.1, field control failure may occur in a few generations depending on the circumstances (Roush and Miller 1986).

The higher Bt resistance gene frequency in Qiuxian County might be associated with high initial resistance allele frequency. Similar resistance allele frequency (0.033 in 2000 and 0.0685 in 2001) was detected in another county within the same province (Shen et al. 2004). Although Bt cotton was first introduced in 1998 in Qiuxian County, Bt sprays had been applied for controlling cotton bollworm since 1991 (He et al. 2001). F_2 screen performed in 1999 indicated that the initial resistance allele frequency in *H. armigera* was 0.0058 (95% CI = 0–0.0187) (He et al. 2001), which was beyond the criterion ($<10^{-3}$) for refuge strategy to work effectively (Roush 1994). The populations had already become resistant to Bt sprays in the early 1990s (Shen and Wu 1995) before Bt cotton was introduced in the region. It was likely that the resistant population became cross-resistant to Bt cotton when it replaced Bt sprays in 1998.

Large-scale adoption of Bt cotton with single resistant gene cry1Ac against target insects might apply heavy selection pressure and prompted the insects to become adaptive to the single toxin (Cry1Ac) producing cotton (Gould 2003). Since 2001, the region was completely planted with Bt cotton without any conventional cotton serving as refuge. Soybean and peanut became natural (unintentionally planted) refuges for the second and third generations of *H. armigera*, and corn served as the natural refuge in late season (Wu et al. 2004, Wu and Guo 2005). These crops were not as effective refuge crops as non-Bt cotton (Bird and Akhurst 2007). A simulation model for the bollworm adaptation in northern China predicted that the insecticidal trait of Bt cotton would be nullified in ≈7–10 yr if Bt cotton proportions 70–100% of the total cotton planting area (Ru et al. 2002). Simulation studies in India also showed that cultivation of 40% of Bt cotton might result in resistant allele frequency increase to 0.5 in 11 yr, which would be likely to cause Bt-cotton failure if no pest control measures were adopted (Kranthi and Kranthi 2004).

In addition, nonhigh-dose Bt cotton may contribute to the resistance evolution in the cotton bollworm. Transgenic Bt cotton varieties planted in the region were NuCOTN33^{B} from Monsanto and various varieties (≈40) from other seed companies in China. All of the varieties expressed single Cry1Ac toxin. Besides this, many farmers reused the seeds harvested from F_1 hybrid or bought Bt cotton seeds from unregistered seed providers for low price. Therefore, not all of the cotton varieties were confirmed to produce high enough insecticidal protein to kill all individuals. Some heterozygotes with low-level resistance could survive on these low Bt expression cotton varieties, and then it allowed resistant allele(s) to accumulate in the field population. Therefore, potential low-dose Bt cotton and lack of effective refuge crops (such as non-Bt cotton) might be a reason for the fast increase of resistant allele(s), and subsequently resulted in an increasing trend of field population since 2001 in the region (data not shown).

Current high-dose plus refuge strategy for delaying resistance evolution relies exclusively on the assumption that the resistant allele is recessive. Insect populations may carry more than one gene involved in mostly recessive or occasionally dominant resistance to a given Cry toxin or even to a set of toxins if cross-resistance occurs (González-Cabrera et al. 2001). Similarly, *H. armigera* may have evolved complex and diverse resistant genetic background in field populations. In Qiuxian County, *H. armigera* produces four generations a year. The first generation feeds on wheat, *Triticum aestivum* L., but the main host plants for the second to fourth generation include cotton, corn, soybean, and peanut. Peanut, soybean, and late-planted corn are all typically planted after wheat in early summer. Adaptability to wide range of host plants allows the insect to expose to a wide range of allelochemicals and subsequently to evolve abilities to detoxify corresponding toxicants (Scriber 2002, Zeng et al. 2007). In this study, we noticed that growth rate and survival rate did not meet exactly as expected for positive and negative lines, or even for known susceptible and resistant strains. Our results indicated that survival rates of some negative lines were higher (more 15%) in F_1 screening, but surviving larvae could not reach the growth rate of the resistant strain. Majority of the surviving larvae from the negative lines had body weight ranging from 0.1 to 0.3 mg. These survivors died eventually after extended selection on Bt cotton for up to seventh day (data not shown). It seemed that there might be many minor Bt resistant alleles in wild males. The minor resistant alleles that each had very small effects on resistance, might be more prevalent in natural populations and may interact with major resistant alleles to accelerate the evolution of resistance (Alstad and Andow 1996). Whether these minor resistant alleles pose a threat to the efficacy of Bt cotton will depend on their fitness costs in the field. Other reasons could be due to variable susceptibilities to Bt toxin protein among individuals or the high tolerance to Bt cotton in field males (Luttrell et al. 1999, Liao et al. 2002); therefore, *H. armigera* does not have to attain RR genotype to overcome the level of Cry1Ac protein produced by transgenic cotton.

In summary, F_1 screen by using a Bt resistant strain and Bt cotton leaves uncovered unusually high resistant allele frequency in Qiuxian County, where conventional cotton was completely replaced by the single-toxin–producing Bt cotton. Long-term use of Bt sprays and Bt cotton and the lack of proper resistance management practice allowed the resistant alleles to build up in field populations of the cotton bollworm. Our results provided alarming caution that rapid resistance development due to a lack of proper resistance management at early stage of Bt cotton implementation might have already caused high dose/

refuge strategy less effective in the region. Therefore, other strategies, such as dual/multi-Bt toxin-producing cotton, biological, chemical, and cultural practices, must be implemented to reduce levels of resistance to Bt cotton in the populations. With a highly Bt-resistant strain available in our laboratory, we will be able to elucidate the molecular and biochemical mechanisms of Bt resistance in this pest. DNA-based detection techniques will be developed to verify whether the field populations have identical or different resistant alleles from the laboratory resistant strain. Inheritance of the resistance, fitness cost, and adaptability to different Bt cotton varieties or Bt toxins will be examined in future studies.

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